Science Journal of Chemistry
2018; 6(5): 77-82
http://www.sciencepublishinggroup.com/j/sjc
doi: 10.11648/j.sjc.20180605.11
ISSN: 2330-0981 (Print); ISSN: 2330-099X (Online)

Antibiofilm Activity of *Lactobacillus* Strains

Ivo Ganchev

Department of General Microbiology, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Email address:
ivotg@abv.bg

To cite this article:
Ivo Ganchev. Antibiofilm Activity of *Lactobacillus* Strains. *Science Journal of Chemistry.* Vol. 6, No. 5, 2018, pp. 77-82.
doi: 10.11648/j.sjc.20180605.11

Received: September 30, 2018; **Accepted**: October 22, 2018; **Published**: November 15, 2018

Abstract: The development of antibiofilm strategies is of major interest in contrasting bacterial biofilms that are a predominant microbial style of life in natural and technical ecosystems. The aim of this study is to evaluate the impact of metabolites produced during the cultivation of lactobacilli in MRS broth, on the biofilm-formation activity of co-cultures *Bacillus subtilis* and *Escherichia coli* K-12 strains. For this purpose, several classical microbiological tools, in particular method for static cultivating of biofilms in 96-well polystyrene plates, and confocal laser scanning microscopy were applied. Thus, the inhibitory effect of eight *Lactobacillus* strains, isolated from homemade dairy products manufactured in Rodopi Mountain, Bulgaria, has been estimated. A strain-specific anti-biofilm activity of cells-free supernatants from eight exponential *Lactobacillus* cultures on the biofilms formed by *Bacillus subtilis* NBIMCC 170 and *Escherichia coli* K12 strain 1655 and by co-culture of *B. subtilis* NBIMCC 168 - *E.coli* K12- 1655 was observed. *Lactobacillus plantarum* L32 strains exhibited a good antibiofilm activity against co-cultures of *Bacillus subtilis* and *Escherichia coli* K12 strains. Data shows that the cell-free supernatants of *Lactobacillus delbrueckii subsp. bulgaricus* strain stimulate sporulation process in the structure of the biofilm by *B.subtilis* 170 and *E.coli* K-12 1655 strains and by *B. subtilis* 168 and *E.coli* K-12 1655 strains in comparison to *Lactobacillus plantarum* strains. In the structure of formed biofilms, the role of dominant species is implemented by *B. subtilis* strains in the presence of cell-free supernatants of *Lactobacillus* strains and at delution rate of cell-free supernatants of *Lactobacillus plantarum* L32 strains in MRS broth in the range from 1:10 to 1:1000. The data of the confocal laser scanning microscopy shows that at dilution rate of cell-free supernatant of 10^{-1} leads to appearance of blank optical field, the increase of metabotnite products of *Lactobacillus plantarum* L32 strain at dilution rate in the range of 10^{-2} – 10^{-3} creates conditions for increasing of intensity of staining by immunofluorescence days in this study. The obtained results showed that a strong anti-biofilm forming effect was obtained with *Lactobacillus plantarum* L32 culture in MRS broth.

Keywords: Biofilms, *Lactobacillus* Strains, Anti-Biofilm Activity, *B. Subtilis*, *E. coli* K12 1655

1. Introduction

The microorganisms are often prevalent in natural ecosystems in the form of the specific organized structures called biofilms. The biofilms are communities of microbial species that are attached on various biotic and abiotic surfaces [1, 2]. These consortia are formed by cells of different microbial species [3] consist of microbial cells and a wide range of self-generated extracellular polymeric substances (EPS), including polysaccharides, nucleic acids, and proteins. [2, 4]. The biofilms are attached on different, according to their nature and structure substrates, from which the microbial cells derive their nutrients [2, 5, 6] This form of co-existence of microorganisms ensures the resistance of cells against the effects of high temperatures, osmotic pressure, high salt content and the low pH of the medium [2, 7]. However, in a number of industrial processes and in the pathogenesis of serious diseases formation of biofilms has a negative effect.

Different agents have been applied to inhibit the growth and development of microbial biofilms [1].

The probiotics (*pro*-for and *bios* - life) are live microorganisms, which when administered in adequate amounts confer a health benefit on the host [7]. Lactobacilli and bifidobacteria are widely accepted as probiotics. Their probiotic activity is based on the antimicrobial metabolites formation [8-10] competition for nutrients and competition adhesion on epithelial layer in the intestinal tract [11, 12], on the modulation of immune response and the ability to prevent

biofilm forming [13, 14]. The information concerning modulation of the biofilms, formed by various microbial species, remains scarce in modern scientific literature [15].

With this aim the *in vitro* effect of the *Lactobacillus* metabolites, isolated from homemade dairy products manufacturexd in Rodopi Mountain, Bulgaria, and produced in MRS broth, on the biofilms of the co-cultures *Bacillus subtilis* - *Escherichia coli* was estimated.

2. Materials and Methods

2.1. Bacterial Strains

B. subtilis 168, *B. subtilis* 170 (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria) and *E. coli* 1655 from the collection of Institute of Molecular Biotechnology, Jena, Germany were used in the present study. All strains were inoculated into 9 ml of liquid culture medium Luria Bertrani (LB) broth and incubated for 18 hours at 37°C before the beginning of each determination. The *Lactobacillus* strains (*Lactobacillus delbrueckii subsp. bulgaricus*, *Lactobacillus plantarum* L22, *Lactobacillus plantarum* L31, *Lactobacillus plantarum* L46, *Lactobacillus plantarum* L21, *Lactobacillus plantarum* L32, *Lactobacillus plantarum* L47, *Lactobacillus plantarum* L33) have been isolated from homemade fermented milk products (Laboratory "Genetics of lactic acid bacteria and probiotics" at the Institute of Microbiology, BAS). For the purpose of experimental, they were cultured twice (for 24 hours at 37°C under anaerobic conditions) in a medium MRS (Merck) broth (pH 6.5). Cells-free supernatants from exponential *Lactobacilus* cultures are prepared by centrifugation (6000 rpm, Hereus) and subsequent filtration (0.22 μm, Millipore). Sterile cells-free filtrates (fCFS) stored at -20°C to the beginning of the definition.

2.2. Method of Static Cultivating of Biofilms

The effect of metabolic products by strains of *Lactobacillus* on the growth of the biofilm by mixed population of *B. subtilis* 170 and *B. subtilis* 168 which *E. coli* strains is found after culturing them in medium M63 (0,02 M KH_2PO_4, 0,04 M K_2HPO_4, 0,02 M $(NH_4)_2SO_4$, 0,1 mM $MgSO_4$ and 0,5% glucose) containing or not containing (control) supernatant of MRS broth at a dilution of 1:10, 1:100 and 1:1000. The mixed population of strains of *B. subtilis* and *E. coli* species was cultured in LB broth for a time of 24 hours at a temperature of 37°C, at a diluted ratio of 1: 100 in M63 with added fCFSs (10% v/v) from *Lactobacillus* cultures in MRS broth at a dilution of 1:10, 1:100 and 1:1000 against control - M63 medium with 5% v/v MRS broth only.

2.3. Determination of Biomass Growth of Biofilms

After cultivation at 20°C for 24 h from each well was separated carefully the culture medium. After three times washing with sterile saline solution, a staining of biofilms in half of the wells was carried out with 200 μl 0,1% v/v solution of crystal violet (CV) over a period of 15 min, gently release and washing of dye in each well, then were fixed 200 μl of 70% ethanol. Following 15 min incubation at RT the optical density (OD) at 570 nm was measured on "ELLIZA Reader ELTA 90".

2.4. Determining of Number of Living Cells (CFU) in the Biofilms

In the second half of the wells were added 200 μl 0.85% sodium chloride solution. Using a sterile knife biofilm was separated carefully. Each variant were made in 6 wells. Solutions with a release biofilm were collected in sterile Eppendorf tubes. The number of living bacteria (CFU/ml) in the biofilms was determined, according to the method of Koch [15], after inoculation on Meat Peptone Agar (NCIPD-Bull Bio, Sofia, Bulgaria) to estimate CFU of *B. subtillis* and on MacConkey agar (NCIPD-Bull Bio, Sofia, Bulgaria) for counting of number of *E. coli* 1655 strain.

2.5. Motility Assay

Motility was determined after culture of mixed cultures on medium M63 containing agar (0.3% w/v) with added 1% (v/v) sterile filtered supueranatants of *Lactobacillus* strains under control M63 agar without supueranatants. After incubation for 24 h at 20°C is reported the diameter of the zone of motility.

2.6. Microscope Assay

After inoculation of the cultures in a medium M63 with or without added supernatants of lactic acid bacteria, suspension was placed on a pre-autoclaving slides and incubated at 20°C for a period of 24 h. Immediately after that it was carefully separated liquid medium and perform complex staining with dye Live Bacterial Gram Stain Kit - CF™594 conjugate of wheat germ agglutinin (WGA) and DAPI and CongoRed, according to protocol of BIOTIUM company. The observations were carried out using confocal laser scanning microscope Leica TCS SPE at wavenlenth at 540 nm.

2.7. Statistical Treatment

All experiments were performed three times, presented results represent the average of three independent each other determinations. It was used a Student test, the differences of individual values were considered statistically significant at $P < 0.05$.

3. Results and Discussion

The ability of seven *Lactobacillus plantarum* strains and one *Lactobacillus bulgaricus* strain to inhibite biofilm formation process was studied. They have been selected among isolates from homemade dairy products evaluated as candidate probiotics in previous studies [16]. The highest sensitivity of the biofilm by combined populations of strains was established in the presence of metabolites secreted by *Lactobacillus plantarum* L32 strain ($p < 0,05$) (Figure 1).

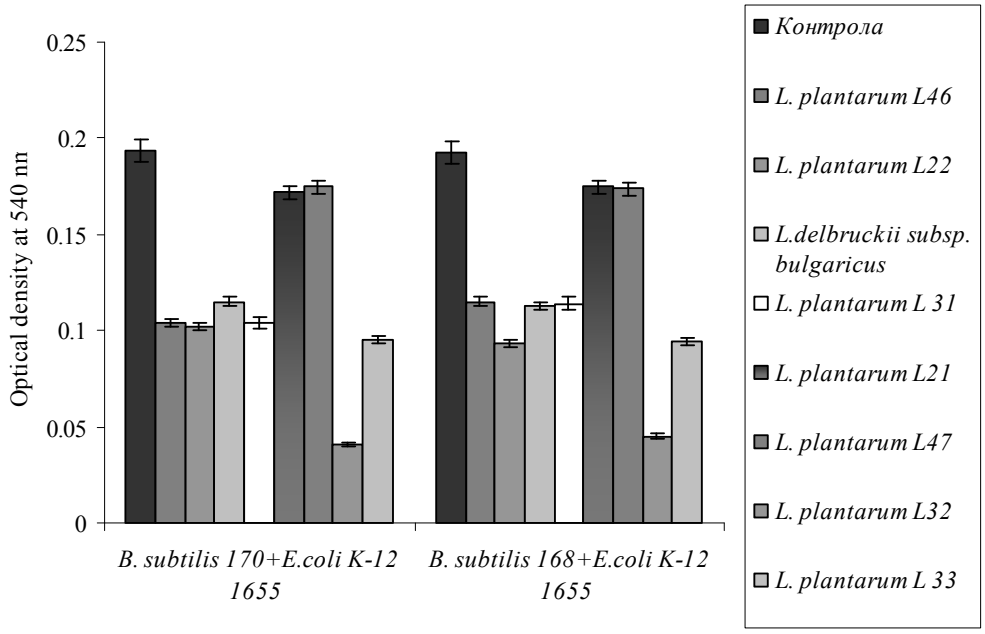


Figure 1. *Effect of supernatants of lactic acid bacteria on biomass of mixed biofilms formed in microplates in co-cultivation at 20°C for 24 h in medium M63 with supernatants of lactic acid bacteria at dilution rate of 1:10 or without supernatants (controls).*

Under these culture conditions the biofilm is observed a strong decrease of the optical density at 540 nm from an 0,194 ± 0,003 to 0,041 ± 0,002 for *B. subtilis* 170 and *E. coli* K-12 1655 strains ($p<0,05$) and 0,193 ± 0,001 to 0,045 ± 0,003 for *B. subtilis* 168 and E. coli K12-1655 strains ($p<0,05$). With the weak antibiofilm forming ability differ metabolic products formed by *Lactobacillus plantarum* L21 and *Lactobacillus plantarum* L47 strains ($p <0,001$). *Lactobacillus delbrueckii subsp. bulgaricus* strain also has a high anti-biofilm-forming ability as well as in the mixed population of *B. subtilis* 170 and *E. coli* K-12 1655, and in the pair of *B. subtilis* strains 168 and *E. coli* K-12 1655. But in the comparation to *Lactobacillus plantarum* L32 strain is less pronounced (Figure 1).

The results correlate with the diameter of area under cultivation of mixed microbial populations on a medium containing 0.3% agar (w/v) to assess of mobility under the influence of metabolites by a culture of *Lactobacillus plantarum* L32, the diameter of the zone was reached to a value of 0,05 cm for pair studied strains (Figure 2).

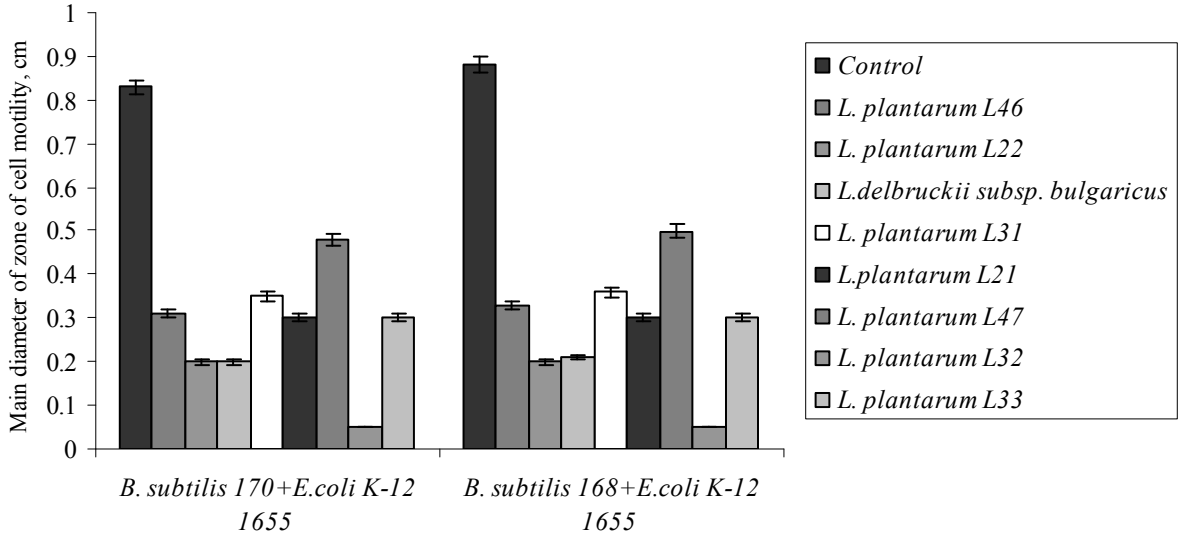


Figure 2. *Influence of supernatants of lactic acid bacteria on the diameter of the zone of cell motility in co-cultivation at 20°C for 24 h in medium M63 with 0,3% agar with supernatants of lactic acid bacteria at dilution rate of 1:10 or without supernatants (controls).*

The active metabolites of *L. delbrueckii subsp. bulgaricus* strain in a medium MRS determine a relatively uniform distribution of the populations of strains of *E. coli* and *B. subtilis* species in the structure of the biofilm, which represents 0,45% of the control samples (Table 1, Table 2). Strong reduction of the population of *B. subtilis* 170 and *B. subtilis* 168 strains in association with *E. coli* K-12 1655 strain is observed in the presence of metabolites of

Lactobacillus plantarum L33, *L. plantarum* L22, *L., plantarum* L31, *L. plantarum* L46 strains, while the *E. coli* K-12 1655 strain exhibits highest sensitivity to effects of *CFSs from L. plantarum*.

Data shows that some products from *Lactobacillus delbrueckii subsp. bulgaricus* culture stimulate sporulation process in the structure of the biofilm by *B. subtilis* 170 and *E. coli* K-12 1655 strains and by *B. subtilis* 168 and *E. coli* K-12 1655 strains in comparison to *Lactobacillus plantarum* strains (Table 1, Table 2). In the presence of spent cultures (1% v/v) of *L. plantarum* L21, *L. plantarum* L47, *L. plantarum* L32, *L. plantarum* L33 strains does prevents the spores-forming in the structure of biofilms (Table 1, Table 2). Moreover the effects were dose-dependent.

Decreasing of activity of supernatant of lactic acid bacteria is accompanied by increase in the biomass of biofilms under static cultivation in the present study, the optimal density was varied from 0 to 0,182 ± 0,003 for of *B. subtilis* 170 and *E. coli* K12 -1655 strains and 0,183 ± 0,002 in *B. subtilis* 168 and *E. coli* K-12 1655 with an increase of dilution rate of supernatants of *Lactobacillus plantarum* L32 strain in the range 10^{-1}-10^{-3}. (Figure 4). The process of growth of the biofilm biomass is accompanied by an increase of the diameter of the zone of cell motility, it was established a linear relationship between the sampled pairs of strains (Figure 5).

Culturing in medium with glucose content of 0,5% results in the appearance of the biofilms with flat topology characterized by high surface coverage (Figure 3). At degree of dilution of supernatant of 10^{-1} leads to appearance of blank optical field, the increase of metabotnite products of *Lactobacillus plantarum* L32 strain at dilution rate in the range of 10^{-2} – 10^{-3} creates conditions for increasing of intensity of staining by CongoRed and WGA. At this conditions it was appeared biofilms irregular topology characterized by low surface coverage for all tested pair strains in the study.

Table 1. *Influence of supernatants of the Lactobacillus strains on the structure of the biofilm community of B. subtilis 170 and E. coli K-12 1655 strains.*

№	Strain	Number in multispecies biofilms, cfu/ml		Spores cfu/ml
		***B. subtilis* 170**	***E. coli* K-12 1655**	
1.	Control	$(15,3\pm0,16).10^6$	$(14,2\pm0,10).10^6$	$(5,8\pm0,2).\ 10^2$
2.	*Lactobacillus plantarum* L46	$(4,66\pm0,2).10^4$	$(1,2\pm0,1).\ 10^4$	$(0,8\pm0,0).\ 10^2$
3.	*Lactobacillus plantarum* L22	$(4,2\pm0,2).10^4$	$(1,86\pm0,4).\ 10^4$	$(0,3\pm0,0).\ 10^2$
4.	*Lactobacillus delbrueckii subsp. bulgaricus*	$(4,53\pm0,2).10^4$	$(4,8\pm0,5).\ 10^4$	$(2,6\pm0,0).\ 10^2$
5.	*Lactobacillus plantarum* L31	$(4,8\pm0,3).10^4$	$(1,4\pm0,2).\ 10^4$	$(0,4\pm0,0).\ 10^2$
6.	*Lactobacillus plantarum* L21	$(15,26\pm0,3).10^4$	$(0,4\pm0,2).\ 10^4$	0
7.	*Lactobacillus plantarum* L47	$(22,6\pm0,4).10^4$	$(3,0\pm0,2).\ 10^4$	0
8.	*Lactobacillus plantarum* L32	0	0	0
9.	*Lactobacillus plantarum* L33	$(6,26\pm0,3).10^4$	0	0

Table 2. *Influence of supernatants of the Lactobacillus strains on the structure of the biofilm community of B. subtilis 168 and E. coli K-12 1655 strains.*

№	Strain	Number in multispecies biofilms, cfu/ml		Spores cfu/ml
		B. subtilis 168	**E. coli K-12 1655**	
1.	Control	$(10,7\pm0,25).10^6$	$(10,36\pm0,32).10^6$	$(5,4\pm0,6).\ 10^2$
2.	Lactobacillus plantarum L 46	$(4,3\pm0,3).10^4$	$(1,33\pm0,2).\ 10^4$	$(0,7\pm0,0).\ 10^2$
3.	Lactobacillus plantarum L22	$(4,66\pm0,3).10^4$	$(1,26\pm0,5).\ 10^4$	$(0,2\pm0,0).\ 10^2$
4.	Lactobacillus delbrueckii subsp. bulgaricus	$(4,8\pm0,3).10^4$	$(4,4\pm0,4).\ 10^4$	$(2,8\pm0,0).\ 10^2$
5.	Lactobacillus plantarum L31	$(4,06\pm0,11).10^4$	$(1,27\pm0,3).\ 10^4$	$(0,4\pm0,0).\ 10^2$
6.	Lactobacillus plantarum L21	$(11,2\pm0,2).10^4$	$(0,4\pm0,1).\ 10^4$	$(1,2\pm0,0).\ 10^2$
7.	Lactobacillus plantarum L47	$(21,2\pm0,4).10^4$	$(3,6\pm0,2).\ 10^4$	0
8.	Lactobacillus plantarum L32	0	0	0
9.	Lactobacillus plantarum L33	$(6,86\pm0,4).10^4$	0	0

Table 3. *Influence the degree of dilution supurantant of Lactobacillus plantarum L32 strain on the number of colonies in the structure of biofilms by B. subtilis 170 and E. coli K-12 1655 strains and spores.*

№	Dilution rate	Number in multispecies biofilms, cfu/ml		Spores cfu/ml
		B. subtilis 170	**E. coli K-12 1655**	
1.	Control	$(15,3\pm0,16).10^6$	$(14,2\pm0,10).10^6$	$(5,8\pm0,2).\ 10^2$
2.	10^{-1}	0	0	0
3.	10^{-2}	$(1,66\pm0,11).10^4$	$(0,2\pm0,0).\ 10^4$	$(0,2\pm0,0).\ 10^2$
4.	10^{-3}	$(8,3\pm0,30).10^4$	$(0,2\pm0,0).\ 10^4$	$(0,4\pm0,0).\ 10^2$

The reduction of metabolic products, which was achieved in this study by increasing the degree of dilution is accompanied by a proportional increase in the number of populations in mixed biofilms for all pair strains in which the role of dominant species is implemented by strains of *B . subtilis* species (Table 3, Table 4). The number of colonies into the structure of biofilms reaches values of (8,3 ± 0,30).10^4 cfu/ml for *B. subtilis* 170 and *E. coli* K-12 1655 strains and (5,6 ± 0,52) x 10^4 cfu/ml for *B.subtilis* 168 and *E. coli* K-12 1655 strains at dilution rate of 10^{-3}, which is many times higher of the number of *E. coli* K-12 1655 strains. The impact of diluted supernatants of vaginal and isolated from

dairy products strains of *Lactobacillus* genus on the growth of biofilms of strains *E. coli* K-12 was investigated in the study of Vacheva et al [17]. The dilution rate of 10^{-2} did not have a significant impact on the growth of biofilms of *E. coli* K-12 strains. In this biofilm modulating ability is determined by the composition of the supernatant and characteristics of the strains. Some strains of *E. coli* species are characterized by the ability to respond to metabolites of lactic acid bacteria by descent of cell hydrophobicity and mobility during biofilm formation. On the other hand, the process of biofilm formation of *E. coli* K-12 strains is inhibited by protein factors, as evidenced by data with the impact of proteinase K [17]. A similar mechanism of action of *Lactobacillus plantarum* L32 strain obviously explains results obtained in this study associated with the formation of biofilms by mixed microbial population.

The obtained values are in close relation with the induction process of sporulation, the total number of spores in mixed biofilms reaches the value of (0,4 ± 0,0). 10^2 cfu/ml for *B. subtilis* 170 and *E. coli* K-12 1655 strains, which is approximately two times more than the value for *B. subtilis* 168 and *E. coli* K-12 1655 strains. The percentage of spores in the structure of biofilms varied from 0.02 % for *B. subtilis* 170 and *E. coli* K-12 1655 strains to 0.2% for *B. subtilis* 168 and *E. coli* K-12 1655 strains, which confirms the conclusion of the study of Hamon and Lazazzera [18] that the sporulation is not an essential condition for development of biofilms by *B. subtilis* strains. A similar results were seen in their association with *E. coli* K-12 1655 strain in the presence of metabolites produced by *L. plantarum* L32 strain in the present study.

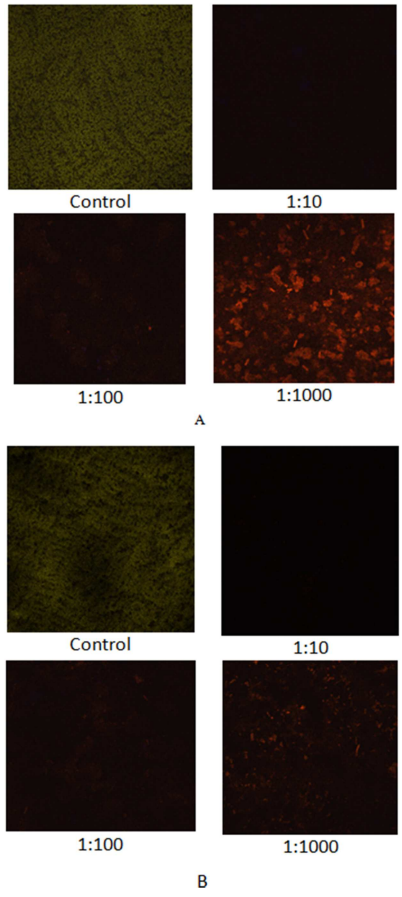


Figure 3. *Influence of the degree of dilution of supernatants of Lactobacillus plantarum L32 on morphological features of biofilms by: B. subtilis 170 u E. coli K-12 1655 /A/, B. subtilis 168 u E. coli K-12 1655 /B/.*

Table 4. *Influence the degree of dilution of supurantants of Lactobacillus plantarum L32 strain on the number of colonies in the structure of biofilms by B. subtilis 168 and E. coli K-12 1655 strains and spores.*

№	Dilution rate	Number in multispecies biofilms, CFU/ml		Spores cfu/ml
		B. subtilis 168	E. coli K-12 1655	
1.	Control	$(10,7\pm0,25).10^6$	$(10,36\pm0,32).10^6$	$(5,4\pm0,6).\ 10^2$
2.	10^{-1}	0	0	0
3.	10^{-2}	$(0,6\pm0,00).10^4$	$(0,4\pm0,0).\ 10^4$	$(0,8\pm0,0).\ 10^2$
4.	10^{-3}	$(5,6\pm0,52).10^4$	$(0,6\pm0,0).\ 10^4$	$(1,8\pm0,0).\ 10^2$

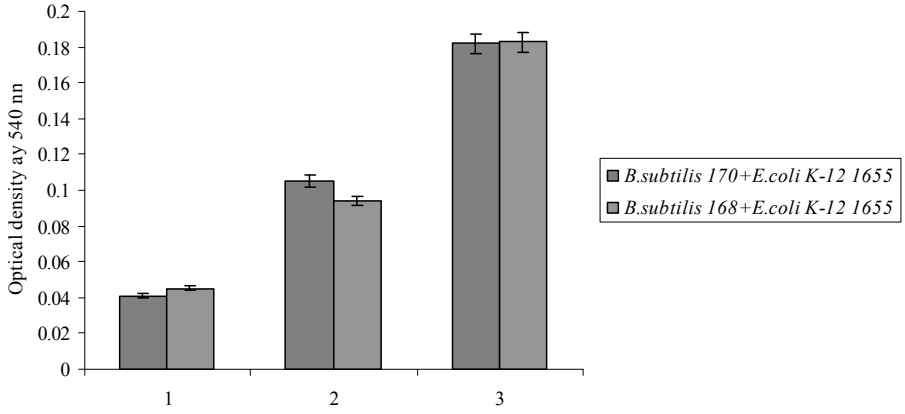
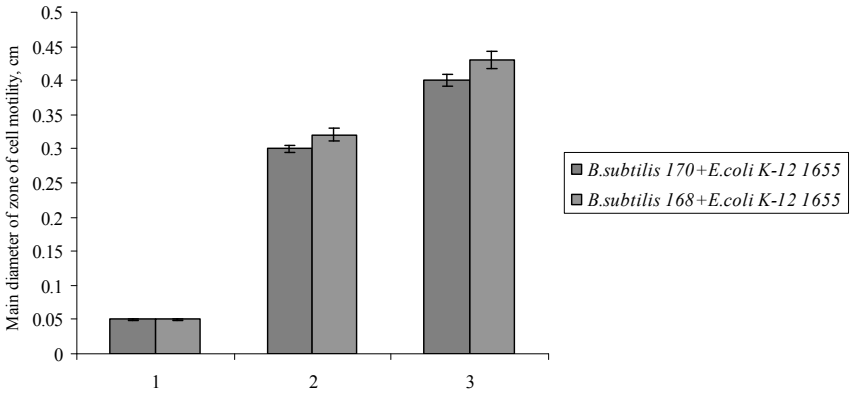


Figure 4. *Effect of the dilution of the supernatants of Lactobacillus plantarum L32 strain on biomass of biofilms: 1. 1:10; 2. 1:100; 3. 1:1000.*

y=0,0705. x-0,0317, r^2= 0.9972 - *B. subtilis* 170 и *E. coli* K-12 1655.
y=0,069. x-0.0303, r^2= 0,9753 - *B. subtilis* 168 и *E. coli* K-12 1655.



y=0,175. x-0,1, r^2=0.9423 - *B. subtilis* 170 и *E. coli* K-12 1655, *B. subtilis* 168 и *E. coli* K-12 165

Figure 5. *Influence of the degree of dilution of supernatants of Lactobacillus plantarum 32 on the diameter of the zone on medium of 0,3 % agar: 1. 1:10; 2. 1:100; 3. 1:1000.*

4. Conclusions

The influence of supernatants of lactic acid bacteria strains on biofilm formation by mixed populations of *B. subtilis* 170 and *E. coli* K-12 1655, and *B. subtilis* 168 and *E. coli* K-12 1655 strains was investigated. With the strong anti-biofilm forming effect are characterized the supernatants of exponential culture of *Lactobacillus plantarum* L32 strain cultivated in medium MRS. The increase of degree of their dilution results in an increase of biomass of biofilm, stimulates the appearance of cell motility, induced sporulation in structures of biofilms.

Acknowledgements

The author is grateful Assoc. Prof. Svetla Danova from Institute of Microbiology, BAS, Assoc. Prof. Milena Mourjeva from Institute of Biology and Immunology of Reproduction, BAS, and Prof. Markus Maniak from University of Kassel, Germany. This study was support by Institute of Microbiology, BAS, Grant I-11/2014, and The "Erasmus+" Program (Grant TM-10/2016-2017)

References

[1] A. Fariq, A. Saeed, *Curr Microbiol,* 72:489–495 (2016).

[2] P. Kolenbrander, R. Andersen, D. Blehert, P. Egland, J. Foster, R. Palmer. *Microbiology and Molecular Biology Reviews*, 66 (3): 486–505 (2002).

[3] A. Rickard, P. Gilbert, N. J. High, P. Kolenbrander, P. Handley. *Trends in Microbiology*, 11 (2): 94-100 (2003).

[4] M. Lengowski, Maren Witzig, Jens Möhring, Gero M. Seyfang, M. Rodehutscord, *Anaerobe*, 34:132–138 (2015).

[5] A. Rickard, A. J. McBain, R. G. Ledder, P. S. Handley, P. Gilbert. *FEMS Microbiology Letters*, 220: 133-140 (2003).

[6] P. Landini, D. de Antoniani, J. Burgess, R. Nijland. *Appl Microbiol Biotechnology,* 86: 813- 823(2010).

[7] C. Hill et al., *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514 (2014).

[8] G. Dobreva-Yosifova, L. Yocheva, A. Mehmed, S. Danova, S. Antonova-Nikolova, *Biotechnology & Biotechnological Equipment*, 23 (1): 801-805 (2009).

[9] R. Georgieva, L. Yocheva, L. Tserovska, G. Zhelezova, N. Stefanova, A. Atanasova, A. Danguleva, G. Ivanova, N. Karapetkov, N. Rumyan, E. Karaivanova, *Biotechnology & Biotechnological Equipment*, 29(1): 8491 (2015).

[10] M. Petrova, R. Georgieva, S. Dimitonova, N. Ivanovska, N. Hadjieva, Sv. Danova. *Biotechnology & Biotechnological Equipment*, 23: 627-631 (2009).

[11] Z. Dimitrov, I. Gotova, E. Chorbajiska, *Biotechnology & Biotechnological Equipment*, 28 (6): 1079-1083 (2014).

[12] R. Tropcheva, R. Georgieva, S. Danova. *Biotechnology & Biotechnological Equipment*, 25(4): 121-124 (2011).

[13] F. Taheur, B. Kouidhi, K. Fdhila, H. Elabed, R. Slama, K. Mahdouani, A. Bakhrouf, K. Chaieb. *Microbial Pathogenesis*, 97: 213-220 (2016).

[14] A. Rickard, P. Gilbert, N. J. High, P. Kolenbrander, P. Handley. *Trends in Microbiology*, 11 (2): 94-100 (2003).

[15] V. Groudeva, P. Moncheva, S. Naumova, B. Gocheva, T. Nedeva, S. Antonova-Nikolova. Manual of Microbiology, The "St. Kliment Ohridski" University, Sofia, 146-153 (2013).

[16] S. Danova, D. Sc. Thesis, BAS, Sofia, Bulgaria, 2015.

[17] A. Vacheva, R. Georgieva, S. Danova, R. Mihova, M. Marhova, S. Kostadinova, K. Vasileva, M. Bivolarska, S. Stoitsova, *Cent. Eur. J. Biol.*, 7 (2): 219-229 (2012).

[18] M. Hamon, B. A. Lazazzera, *Molecular Microbiology,* 42(5): 1199–1209 (2001).